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14. ABSTRACT

Until recently, 3'end formation was believed to be a static event. The discovery that transformed and rapidly proliferating cells use alternative cleavage and polyadenylation (APA) to shorten the 3'UTR of their mRNAs has important implications in cancer. Truncation of the cyclin D1 mRNA in mantle cell lymphoma (MCL) is one of the earliest reported cases of APA. However, the mechanism that APA is still unknown. The goal of this project is to identify the mechanism of cyclin D1 APA regulation in cancer. So far we have been able to develop dual luciferase plasmids containing the cyclin D1 3'UTR which will enable us to determine the elements important for APA in MCL. In addition, by using RNA Seq. CFIm25 has been identified as an important global regulator of shortening of cyclin D1 mRNA and other genes. The shortened transcripts have been shown to result in increased protein levels resulting in increased cell proliferation, a hallmark of cancer. These data provides a clear link between CFIm25 and regulation of APA and the utility of using novel RNA Seq. technology. This provides a strong research platform for continued research on this project.

15. SUBJECT TERMS
Mantle cell lymphoma, alternative cleavage and polyadenylation, RNA-Seq, cyclin D1

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INTRODUCTION

The oncogene cyclin D1 is important in the disease pathogenesis of Mantle Cell Lymphoma (MCL). It has been shown that cyclin D1 undergoes alternative cleavage and polyadenylation (APA) in MCL through some, yet unknown mechanism. In order to elucidate this mechanism, this project is subdivided into 3 specific aims. The first aim is to identify the cis-elements governing cyclin D1 proximal poly (A) site selection in MCL. In addition I will determine the role of CFIm25 and CFIIm-Pcf11 in APA of cyclin D1 in MCL. The last aim involves the global analysis of genes in MCL cancer cells whose pPAS usage is regulated by CFIm-25 and CFIIm-Pcf11. This first year progress report will describe the progress made so for specific subsections of all three aims. In brief, we have successfully shown that CFIm25 plays a major role in global regulation of APA in cyclin D1 and other genes. To put the progress made in light of the specific aims into perspective, the following section will provide a brief review on the background in the field.

The role of Cyclin D1 in MCL

Overexpression of the G1-S phase cell cycle regulating oncogene cyclin D1 in MCL occurs as a result of a t(11;14)(q13;q32) chromosomal translocation that places cyclin D1 under the control of B-cell IgH genes transcription enhancers [1]. This overexpression of cyclin D1 contributes to the proliferative signature, which determines the rate of tumor proliferation and patient survival [2]. In addition to the chromosomal translocation, studies have shown that MCL patients that express cyclin D1 with a longer 3'UTR survived longer (3.28 years) than patients who expressed the truncated isoform (1.38 years)[3]. The long and the short cyclin D1 transcripts have the same open reading frame and code for the exact same protein. They only differ in the length of their 3'UTRs [3, 4]. Shortening of the cyclin D1 3'UTR in some MCL patients is due to genomic deletions. In other cases, mutations occur providing a stronger proximal PAS (pPAS) that is then used instead of the more distal PAS (dPAS) [3] through APA. However there are still cases of APA in MCL, which do not involve mutations that generate a stronger pPAS and how the pPAS is selected for use in these cases is still unknown. *Hence the working hypothesis is that in MCL cells, cyclin D1 pPAS selection is regulated by cis elements adjacent to it.* This is investigated in Aim 1.

Alternative Cleavage and Polyadenylation (APA)

Similar to what is seen in cyclin D1, most cases of alternative cleavage and polyadenylation(APA) involve using alternative polyadenylation signals (PAS) within the same terminal exon [5]. Usage of the pPAS results in shortening of the 3'UTR may result in the elimination of destabilizing elements including miRNA binding sites and AU-rich destabilizing elements (Figure 1). The resulting truncated mRNA transcripts are more stable resulting in increased translation [6]. Shortening of the 3'UTR is widespread and has been observed in rapidly proliferating cells [7] and has been implicated in tumorigenesis [6, 8]. Ongoing work in the field seeks to identify all the factors that regulate APA in cancer. *We hypothesized that alterations in the levels of 3'end cleavage and polyadenylation factors determine PAS site choice.* This is the goal of Aim 2.

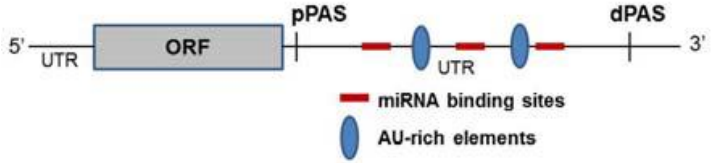


Figure 1: Use of the pPAS instead of the dPAS results in elimination of 3'UTR destabilizing elements.

Identification of genes in MCL that undergo APA

Since the discovery of several oncogenes that undergo APA in cancer by Mayr *et al* using Northern blots [6], next generation sequencing has been extensively used to identify APA changes on a global scale. Besides cyclin D1, the genes that undergo shortening in MCL are still unknown. Preliminary studies in our lab led us to *hypothesize that the there are other genes undergoing APA besides cyclin D1in MCL cancer cells*. This will be the focus of Aim 3.

BODY

Both the negative and positive outcomes for this first year of research will be reported in this section. Research for three aims were performed concurrently and the results will be presented for each specific aim and linked to the task listed in the Statement of Work (SOW).

Specific Aim 1: Identify the cis-elements governing cyclin D1 proximal poly (A) site selection in MCL.

Aim 1 a and b. Clone cyclin D1 upstream of EGFP in pcDNA3.1 plasmid and check for expression. Perform site directed mutagenesis to eliminate any stop codons and within Cyclin D1.

Although EGFP had already been cloned into pcDNA3.1, the plasmid did not have a tag for checking expression, so efforts were made to clone a fragment of the 3'UTR (~300nts) of cyclin D1 sequence downstream of the Myc-tagged GFP expressing plasmid pcDNA4 instead. The expected results were that if the cyclin D1 proximal PAS is used, there will be no expression of GFP. However if the GFP PAS is used instead, there will be GFP expression (Figure 2, left panel). The cherry plasmid was co-transfected with the pcDNA4

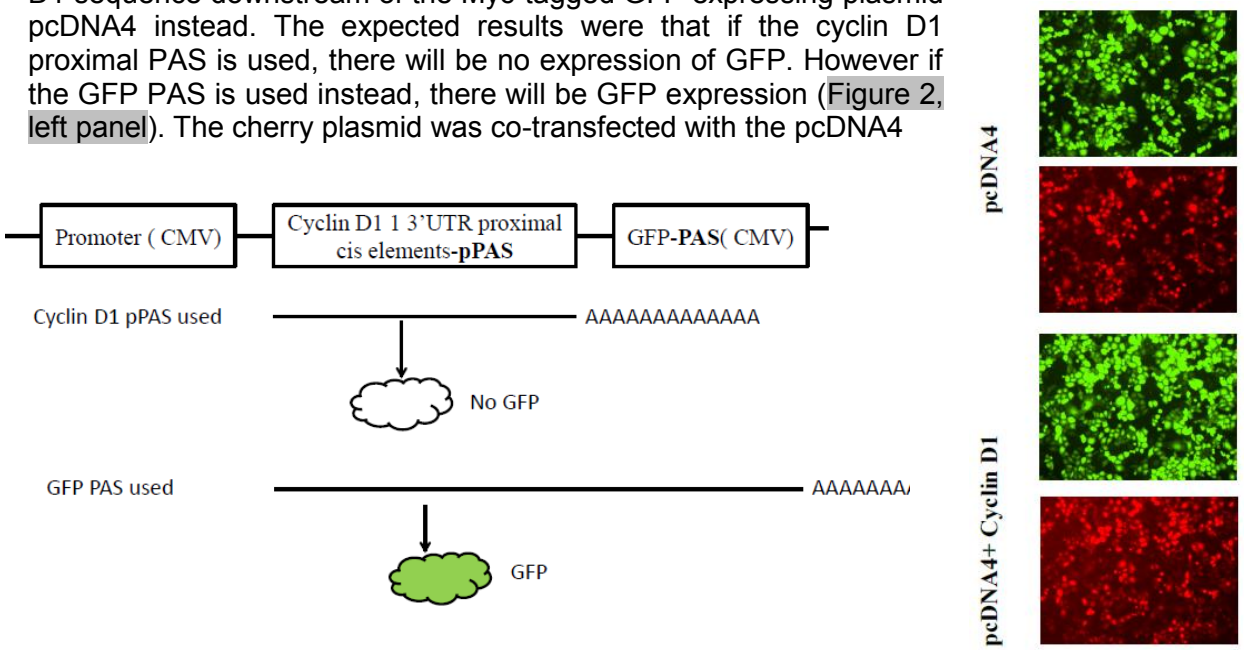


Figure 2. Left panel. The reporter system developed to detect usage of cyclin D1's proximal poly A site (pPAS). Right panel. Immunofluorescence data after cells were transfected with the pcDNA4 GFP plasmid alone or the pcDNA4 plasmid with the cyclin D1 3'UTR upstream of GFP. Either plasmid was co-transfected with cherry plasmid for use as a transfection control.

plasmid to correct for transfection efficiency. As shown (Figure 2, right panel) Cyclin D1 was successfully cloned in frame with GFP and we can still detect GFP expression. However, this reporter system did not attenuate GFP signal as was expected, i.e. the cyclin D1 pPAS was not used. In addition, co-transfection with cherry did not result in a reproducible immunofluorescence signal suggesting that co-transfection may alter the transfection efficiency of each plasmid.

There are a number of possibilities why the cyclin D1 pPAS was not used. It is possible that the cyclin D1 3'UTR fragment used was too short and did not include all the *cis* elements required for proper processing. To address this, 2 longer cyclin D1 3'UTR fragments (both >500bp) were generated (Figure 3) and cloned into the dual luciferase plasmid psi-check 2 (Promega). In this plasmid the cyclin D1 3'UTR plasmid

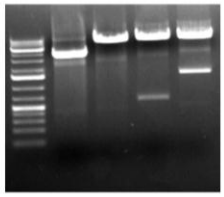


Figure 3. Restriction enzyme digest products showing the 2 different cyclin D1 3'UTR fragments cloned into psi-check.

fragments are cloned downstream of the human Renilla luciferase gene translational stop codon. The advantage of using this plasmid is that it also has human firefly luciferase cloned within the same reporter system allowing for intra-plasmid normalization of transfection eliminating problems observed with co-transfection of 2 different plasmids. This dual cassette reporter system is amenable to detecting effects of RNAi on cyclin D1 half-life which is one of the goals for Specific Aim 2. Since we now have the required clones, we can now proceed with site-directed mutagenesis to identify *cis*- elements and to mimic mutations observed in MCL.

Specific Aim 2: Determine the role of CFIm25 and CFIIm-Pcf11 in APA of cyclin D1 in MCL

MCL is characterized by overexpression of cyclin D1. Here we show that all the MCL cell lines have high levels of cyclin D1 which is undetectable in the normal B-cell line RPMI 1788 (Figure 4).

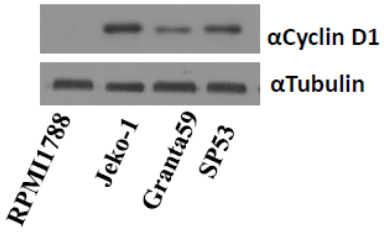


Figure 4. Western Blot analysis of normal B cell (RPMI 1788) and MCL cell lysates.

Aim 2a. Optimize RNAi in Jeko-1 and RPMI 1788

In this aim the goal is to knock down CFIm25 and Pcf11 in RPMI 1788 normal B-cells and in the MCL cell line Jeko-1. MCL cells have been reported to be extremely difficult to transfect. In our lab we have developed a Lipofectamine based RNAi protocol that has worked in most of the cell lines we have tested so far, with a very high degree of knockdown achieved. Here we show that when we used two different siRNAs against CFIm25 in HeLa and A549 cells we were able to deplete CFIm25 levels (Figure 5a). However, we were unsuccessful in using the same technique to knockdown CFIm25 in RPMI and Jeko-1 (Figure 5b). Efforts are ongoing and in addition to use other transfection reagents we plan to use Nucleofactor, which has been reported in the literature to work on MCL cells.

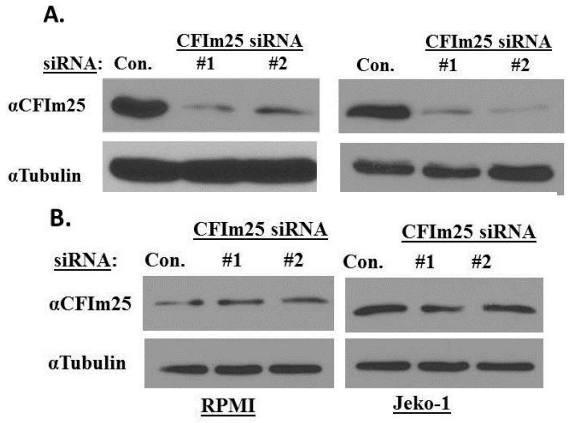


Figure 5. Western blot analysis of lysates after knocking down CFIm25 with 2 different siRNAs (#1 and #2) compared to control (Con.)siRNA.

Aim 2b. Develop RNAi resistant forms of CFIm-25 and CFIIm-Pcf11

One of the goals of this project is to use RNAi resistant plasmids to perform RNAi and rescue experiments. In collaboration with Scott Collum, a graduate student in the lab, we have developed RNAi resistant CFIm25 expression plasmid in Myc-HA-tagged pcDNA3 (Figure 6). One of the genes that underwent 3'UTR shortening after CFIm25 depletion was glutaminase (GLS) resulting in increased protein levels (Figure 6 and Figure 13 right panel). As proof of principle, when CFIm25 siRNA was co-transfected with the CFI25m RNAi resistant plasmid, it was able to abrogate the increase in GLS protein levels (Figure 6).

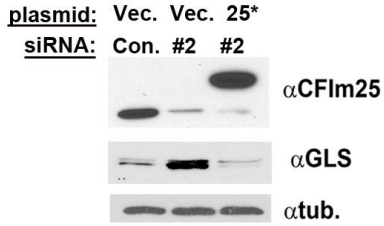
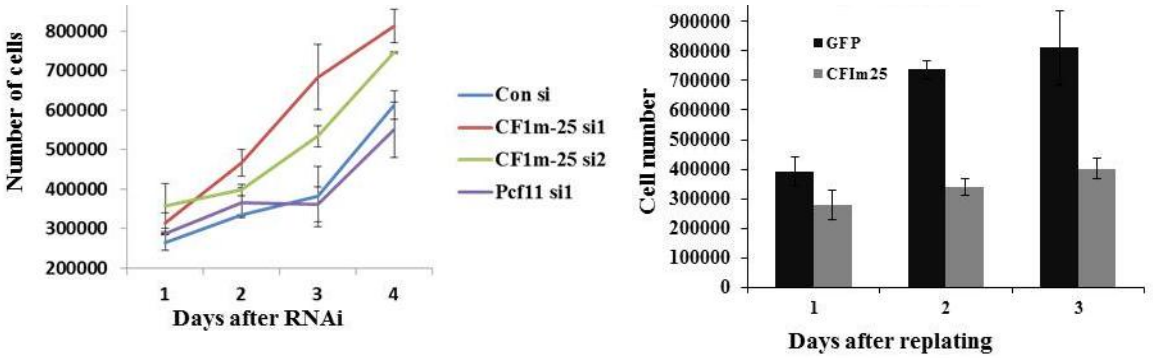


Figure 6. Western of U251 cell lysates after co-transfection of either pcDNA3 GFP (Vec) or RNAi resistant pcDNA3 CFIm25 (25*) with siRNA. The siRNA used was CFIm25 siRNA (#2) or control siRNA (Con.).

Aim 2e. Determine effects of RNAi/overexpression on cell cycle and cell proliferation

Since cyclin D1 drives the G1-S phase of the cell cycle, one of our goals is to investigate the effect of CFIm25 siRNA on cell proliferation in MCL. Preliminary data in HeLa cells shows that



depletion of CFIm25 results in increased cell proliferation (Figure 7). However, the number of cells after depletion of Pcf11 is comparable to the control. When CFIm25 was over-expressed in U251 cells, there was a decrease in cell proliferation.

Figure 7. Left panel. Graph shows effect of different siRNA's on cell growth. Right panel. Graphical representation of the effect of CFIm25 overexpression on cell growth.

Specific Aim 3: Identify genes in MCL cancer cells whose pPAS usage is regulated by CFIm-25 and CFIIm-Pcf11 by using 3'P Seq. Deep sequencing Technology

Aim 3a. Perform 3P-Seq of normal vs. MCL cancer cells

Aim 3c. Perform 3P-Seq after RNAi of cleavage factors in the MCL cell lines

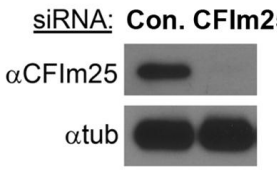


Figure 8. Western blot of HeLa cell lysates after knocking down CFIm25.

Although deep sequencing is not scheduled to be performed until the second year I will report some of the inroads we have made in our pilot studies using this technology. As a proof of principle we initially started using 3P Seq. deep sequencing technology on a sample of HeLa cells. Although we were able to identify the PAS sites used and the cleavage sites, our read density was very low i.e. less than half a million total reads. We then used another deep sequencing technique known as RNA Sequencing (RNA Seq.) which requires less mRNA partitioning and less sample manipulation. I knocked down CFIm25 in HeLa cells (Figure 8) and sent mRNA samples for sequencing at LC Sciences. This RNA-Seq. approach was very successful and resulted in ~150 million reads each for the control and the CFIm25 knockdown (Table 1)

Table 1: Summary of RNA Sequencing data

	# raw reads	# mappable reads	# total mapped reads	Percentile of mapped reads over raw reads
Control	**154,321,468**	**154,105,706**	**114,363,079**	**74.11%**
CFIm25	**146,112,668**	**145,908,590**	**118,211,444**	**80.90%**

A closer look at both the cyclin D1 and Timp2 terminal 3'UTR RNA-Seq. data (Figure 9) shows that there is a decrease in read density in the 3'UTR after CFIm25 depletion similar to previous results we obtained using qRT-PCR primers that allow us to distinguish between distal and proximal PAS usage. This RNA-Seq. data is important because: First it validates our previous qRT-PCR data that CFIm25 depletion results in 3'UTR shortening of cyclin D1 due to increased pPAS usage. Second, any changes in the 3'UTR length can be detected by analysis RNA-Seq data read density.

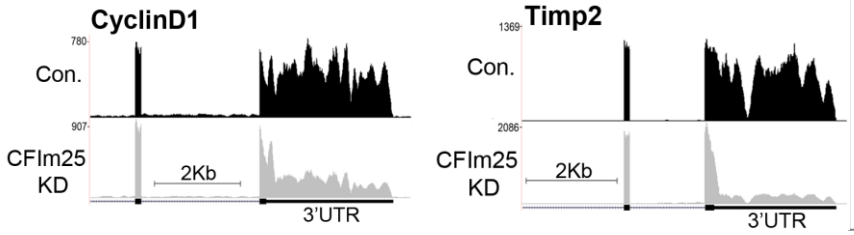


Figure 9. Analysis of the read density map of the last 2 exons of cyclin D1 and Timp-2 from RNA-Seq data. The number of reads is shown on the left for both the control (Con.) and CFIm25 siRNA (CFIm25KD).

Since analysis of the RNA-Seq data by eye is cumbersome and non-quantitative, our lab entered into a collaboration with Dr. Wei Li, a Bioinformaticist from Baylor College of Medicine whose lab specializes in developing complex algorithms to analyze genome wide sequencing data. Dr. Wei Li and his postdoctoral fellow, Dr. Zheng Xia developed a customized algorithm that is able to detect and quantify changes in 3'UTR length between samples. The change in usage of the dPAS between samples is then quantified as the percentage dPAS usage index (ΔPDUI). From this we identified 1002 transcripts that were shortened by usage of the pPAS after CFIm25 depletion, and only 5 transcripts that were lengthened (Figure 10). This suggests that CFIm25 is a global repressor of pPAS usage. The algorithm also allows us to identify novel cases of APA not annotated in any database. Shown here (Figure 11) are Vma21,

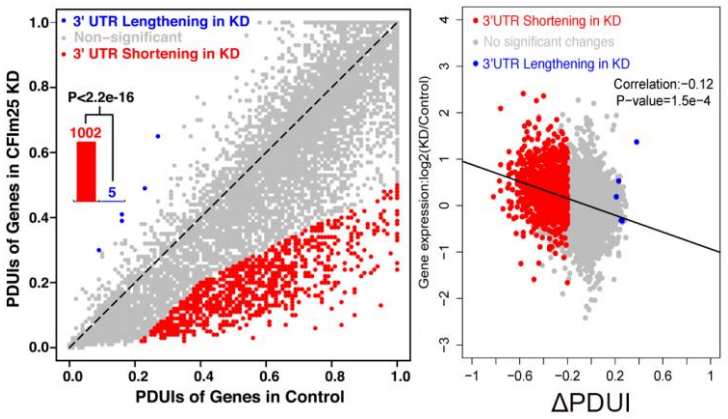


Figure 10. Left panel: Scatterplot and graph (insert) for control and CFIm25 knockdown showing changes in PAS usage on a global scale. Right panel: Shown is a plot of the correlation between usage in dPAS usage and levels of genes after CFIm25 knockdown (KD).

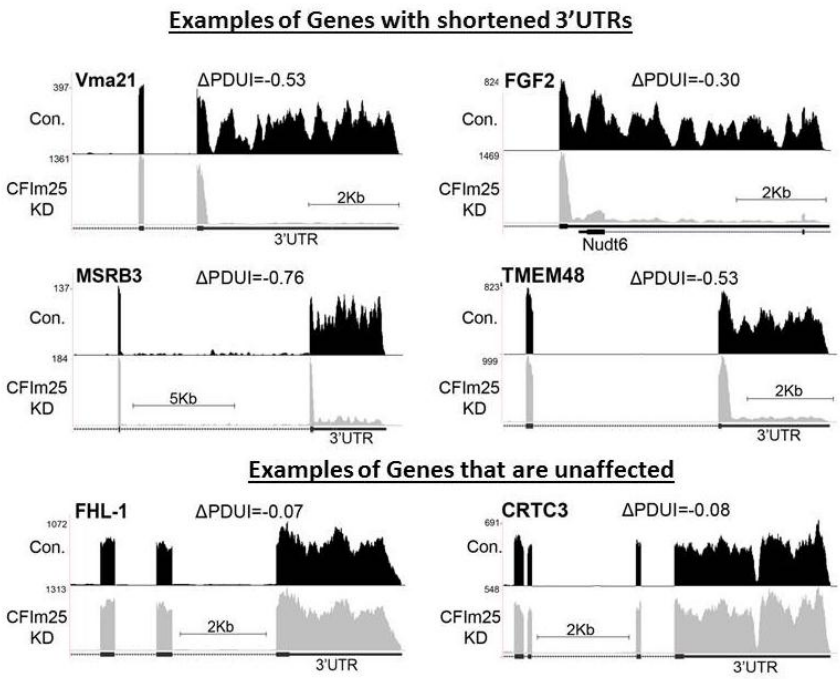


Figure 11. RNA-Seq. read densities of genes that are affected or unaffected by CFIm25 depletion.

FGF2, MSRB3 and TMEM48 which undergo APA after CFIm25 depletion. There are also some genes that are unaffected by loss of CFIm25 despite having long 3'UTRs and the two examples shown are FHL-1 and CRTC3.

Aim 3d. Validate 3P-Seq results on a gene by gene basis using qRT-PCR and map PAS used by 3'RACE.

To validate the results from RNA-Seq. amplicons were designed (Figure 12) for a few select genes to detect changes in distal pPAS usage normalized to total mRNA for each gene.

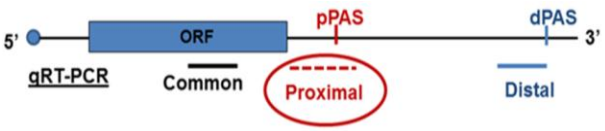


Figure 12. Location of amplicons (common to measure total levels of transcript and distal to detect changes in dPAS usage) used in qRT-PCR.

Quantitative RT-PCR (qRT-PCR) was used to detect APA after CFIm25 depletion using 2 different siRNAs. As shown (Figure 13 , left panel), qRT-PCR was able to validate RNA.Seq. data of 2 genes that were unaltered by CFIm25 depletion and several genes that switched from dPAS to pPAS usage after CFIm25 knockdown. Western blot analysis was then performed to test whether this increase in pPAS usage correlates with increased protein expression. There were significant increase in protein levels for several genes which correlates with a switch to the

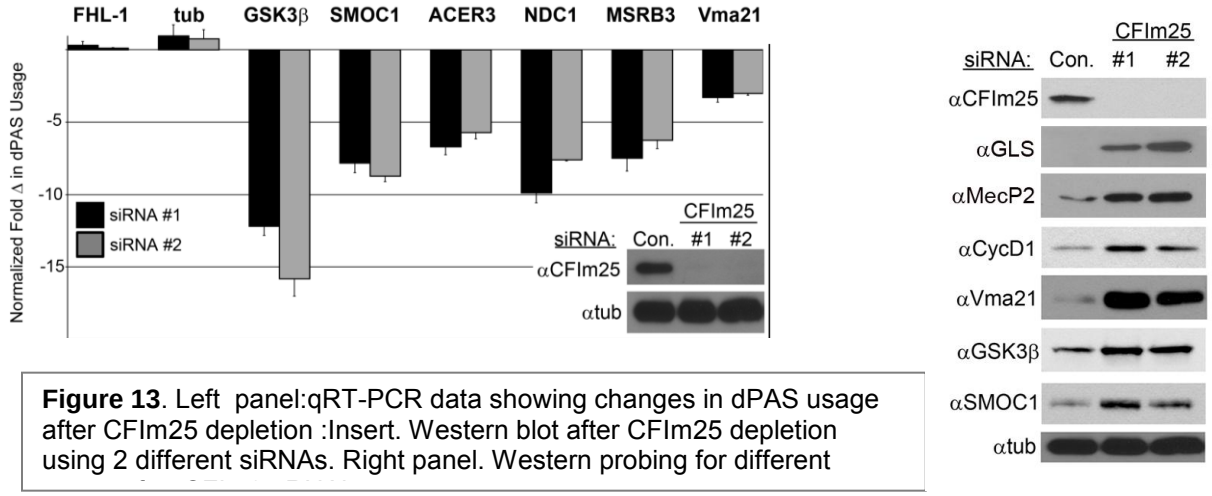


Figure 13. Left panel:qRT-PCR data showing changes in dPAS usage after CFIm25 depletion :Insert. Western blot after CFIm25 depletion using 2 different siRNAs. Right panel. Western probing for different

pPAS resulting in the generation of more stable transcripts. Most notably there were increases seen in levels of glutaminase (GLS), MecP2 and cyclin D1 (Figure 13, right panel) which have been directly associated with increased cell proliferation characteristic of the transformed cell phenotype. This also correlates with increased cell proliferation which was observed in Figure 7. Most notably, we see changes in APA correlating with increased levels of oncogenic cyclin D1, a main driver of MCL pathogenesis.

KEY RESEARCH ACCOMPLISHMENTS

- CFIm25 regulates changes in APA in cyclin D1 and other genes
- The changes from dPAS to pPAS usage regulated by CFIm25 depletion corresponds with a decrease in cell proliferation
- Analysis of RNA Seq. of total mRNA can identify APA changes

REPORTABLE OUTCOMES

Abstracts:

1. Masamha C.P*., Xia Z*., Albrecht T.R., Li W., Shyu A-B., and Wagner, E.J. 2013. RNA 2013 Meeting. 18th Annual Conference. Davos, Switzerland. Title: CFIm25 Links Global change in APA to Cell Growth Control and Glioblastoma Survival. Abstract Number 76-Oral Abstracts. Page 44 (selected competitively for a Plenary Oral Presentation)

Manuscripts:

1. Masamha C.P*., Xia Z*., Albrecht T.R., Li W., Shyu A-B., and Wagner, E.J. 2013. CFIm25 Links Global change in APA to Cell Growth Control and Glioblastoma Survival. Completed Manuscript. (through two rounds of review at *Nature*)

   *Authors contributed equally to this work

Proposed Training:

(Proposed in Aim 3b. Attend class at CSHL on Deep Sequencing Technology)

- Accepted into CSHL training course in Advanced Sequencing Technology and Applications, November 12-24, 2013.

CONCLUSIONS

The work presented here is the progress made within the first year of funding. Cyclin D1 has been successfully cloned into a dual luciferase vector. This will allow for the interrogation of cis elements that are important for APA and enhance our understanding in how cyclin D1 is overexpressed in MCL. The report that CFIm25 can regulate APA on a global scale is very significant because it sheds some light on how APA may be regulated in cancer which is a main focus of current research. The progress made so far will act as the basis for continued research and add greatly to our understanding of APA as a novel method of gene regulation.

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